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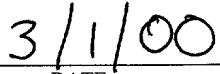
EXPRESS MAIL NO.: EL348123700US  
 ATTORNEY DOCKET NO. 22000.0021U2  
 SERIAL NO.  
 PAGE 1 OF 3 (03/01/00)

CONTINUING APPLICATION TRANSMITTAL FORM (37 C.F.R. § 1.53(b))			
ANTICIPATED CLASSIFICATION OF THIS APPLICATION		PRIORITY APPLICATION: 09/170,754	
CLASS: 435	SUBCLASS: 375.000	EXAMINER: Shuman, J.	ART. UNIT: 1636
<p>To the Assistant Commissioner for Patents:</p> <p>This is a request for filing a continuing application under 37 C.F.R. 1.53(b), of pending prior application Serial No. 09/170,754 filed on October 13, 1998, which is a divisional of Serial No. 09/052,784, filed on March 31, 1998 (now abandoned), which is a continuation of Serial No. 08/258,852, filed June 13, 1994, now U.S. Patent No. 5,807,746, issued September 15, 1998 of Jaeck J. Hawiger and Yao-Zhong Lin for "A NOVEL METHOD FOR IMPORTING BIOLOGICALLY ACTIVE MOLECULES INTO CELLS."</p> <p>If any extension of time is necessary for the filing of this application, including any extension of time necessary in the prior application for maintaining copendency between the prior application and this application, and such extension has not otherwise been requested, Applicant hereby petitions for such an extension in the prior application.</p>			
<p>1. Enclosed is a specification as prescribed by 35 U.S.C. § 112 containing a description pursuant to § 1.71 and at least one claim pursuant to § 1.75, and any drawing required by § 1.81(a).</p> <p>2. The inventor(s) of the invention being claimed in this application is(are): Jaeck J. Hawiger and Yao-Zhong Lin.</p>			
<p>3A. <input checked="" type="checkbox"/> This application is a continuation or divisional application (<u>not</u> a continuation-in-part) that:</p> <p>(i) names as inventors the same or fewer than all of the inventors named in the prior application; and    (ii) contains no matter that would have been new matter in the prior application; and    (iii) a copy of the executed oath or declaration filed in the prior application, showing the signature or an indication thereon that it was signed:</p> <p><input checked="" type="checkbox"/> is enclosed.  <input type="checkbox"/> will follow.</p> <p><input type="checkbox"/> This application is being filed by less than all the inventors named in the prior application. In accordance with 37 C.F.R. § 1.53(d)(4), the Commissioner is requested to delete the name(s) of the following persons who are not inventors of the invention being claimed in this application:</p> <p>--OR--</p>			
<p>3B. <input type="checkbox"/> This application is a continuation-in-part application, or a continuation or divisional application naming an inventor not named in the prior application. A newly executed oath or declaration:</p> <p><input type="checkbox"/> is enclosed.  <input type="checkbox"/> will follow.</p>			
<p>4. <input type="checkbox"/> Amend the Title of the Invention as follows:</p>			
<p>5. <input checked="" type="checkbox"/> Amend the specification by inserting before the first line the sentence:    This application is a continuing application under 37 C.F.R. 1.53(b) of, and claims the benefit of, application serial No. 09/170,754 filed on October 13, 1998, which is a divisional of Serial No. 09/052,784, filed on March 31, 1998, which status is now abandoned, which is a continuation of Serial No. 08/258,852, filed June 13, 1994, now U.S. Patent No. 5,807,746, issued September 15, 1998, which applications and patent are hereby incorporated herein by reference.</p>			
<p>6. <input type="checkbox"/> Priority of foreign application number , filed on in is claimed under 35 U.S.C. 119.  <input type="checkbox"/> The certified copy has been filed in prior application Serial No. , filed on .</p>			
<p>7. <input checked="" type="checkbox"/> Cancel in this application original claims 1-5 and 27-31 of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)</p>			

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8.	<input type="checkbox"/>	A preliminary amendment is enclosed.			
9.	<input type="checkbox"/>	New formal drawings are enclosed.			
10.	<input checked="" type="checkbox"/>	Also enclosed is: Authorization to Treat Reply Requiring Extension of Time as Incorporating Petition for Extension of Time; a copy of the Assignment Recordation Form and the Verified Statement Claiming Small Entity Status, as were filed in Serial No. 08/258,282.			
11.	<input checked="" type="checkbox"/>	The prior application is assigned of record to: Vanderbilt University			
12.	<input checked="" type="checkbox"/>	A verified statement to establish small entity status under 37 C.F.R. 1.9 and 1.27:  [X] is enclosed  [X] was filed in prior application Serial Number 09/170,754 and such status is still proper and desired (37 C.F.R. 1.28(a)).			
13.	The filing fee is calculated as follows:				
TOTAL CLAIMS		22 - 20 =	2	x \$18.00	\$36.00
INDEPENDENT CLAIMS		7 - 3 =	4	x \$78.00	\$312.00
MULTIPLE DEPENDENT CLAIM(S) (IF APPLICABLE) =				+ \$260.00	\$
BASIC FEE					\$760.00
TOTAL OF ABOVE CALCULATIONS =					\$1108.00
REDUCTION BY 1/2 FOR FILING BY SMALL ENTITY (NOTE 37 C.F.R. 1.9, 1.27, 1.28) IF APPLICABLE, AFFIDAVIT MUST BE FILED ALSO.					\$554.00
TOTAL NATIONAL FEE =					\$554.00

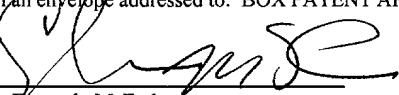
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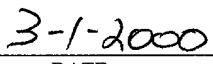
14.	<input checked="" type="checkbox"/>	A check in the amount of \$554.00 is enclosed.
15.	<input type="checkbox"/>	The Commissioner is hereby authorized to charge any fees which may be required under 37 C.F.R. 1.16 and 1.17, or credit any overpayment to Deposit Account No. 14-0629.
16.	<input checked="" type="checkbox"/>	The Power of Attorney in the prior application is to:  a. <input checked="" type="checkbox"/> The Power of Attorney appears in the original papers in the prior application. b. <input type="checkbox"/> Since the Power does not appear in the original papers, a copy of the power in the prior application is enclosed. c. <input checked="" type="checkbox"/> Address all future correspondence to: (May only be completed by applicant, or attorney or agent of record.)
<p>David G. Perryman NEEDLE &amp; ROSENBERG, P.C. Suite 1200, The Candler Building 127 Peachtree Street, N.E. Atlanta, GA 30303-1811</p>		
17.	I hereby verify that all statements made herein of my own knowledge are true; and further that these statements were made with the knowledge that willful false statements and the like are made punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.	
<p>David G. Perryman REG. NO. 33,438</p> <p> SIGNATURE</p> <p> DATE</p>		
<p><input type="checkbox"/> Inventor(s) <input type="checkbox"/> Assignee of complete interest <input checked="" type="checkbox"/> Attorney or agent of record <input type="checkbox"/> Filed under 37 C.F.R. 1.34(a) (Registration No. is acting under 37 C.F.R. 1.34(a):</p>		

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CERTIFICATE OF EXPRESS MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as Express Mail Invoice No. EL348123700US in an envelope addressed to: BOX PATENT APPLICATION, Assistant Commissioner for Patents, Washington, D.C. 20231, on the date shown below.

  
Everardo McFarlane

  
DATE

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Applicant or Patentee: Lin and Hawiger

Attorney's

Serial or Patent No.: 08/258,852

Docket No.: 2200.021

For: "A NOVEL METHOD FOR IMPORTING BIOLOGICALLY ACTIVE MOLECULES INTO CELLS"

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY  
STATUS (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: Vanderbilt University  
ADDRESS OF ORGANIZATION: Nashville, Tennessee 37240

## TYPE OF ORGANIZATION:

University or other institution of higher education

Name of state: Tennessee  
(Citation of statute)

Would qualify as tax exempt under Internal Revenue Service Code (26 USC  
501(a) and 501(c) (3)) if located in The United States of America

Would qualify as nonprofit scientific or educational under statute of state  
of The United States of America if located in The United States of America  
(Name of state)  
(Citation of statute)

SEARCHED  
INDEXED  
SERIALIZED  
FILED

I hereby declare that the nonprofit organization identified above qualifies as a  
 nonprofit organization as defined in 37 CFR 1.9 (e) for purposes of paying reduced fees  
 under section 41(a) and (b) of Title 35, United States Code with regard to the invention  
 entitled "A NOVEL METHOD FOR IMPORTING BIOLOGICALLY ACTIVE MOLECULES INTO CELLS" by  
inventors Yao-Zhong Lin and Jack J. Hawiger described in the specification filed on  
June 13, 1994.

I hereby declare that rights under contract or law have been conveyed to and remain with  
the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual,  
concern or organization having rights to the invention is listed below\* and no rights  
to the invention are held by any person, other than the inventor, who could not qualify  
as a small business concern under 37 CFR 1.9(d) or by any concern which would not  
qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization  
under 37 CFR 1.9(e).

\*NOTE: Separate verified statements are required for each named person, concern or  
organization having rights to the invention averring to their status as small  
entities. (37 CFR 1.27)

NAME N/A

ADDRESS

INDIVIDUAL  SMALL BUSINESS CONCERN  NONPROFIT ORGANIZATION

Page 2 of 2

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Leona Marx  
TITLE IN ORGANIZATION Assistant General Counsel  
ADDRESS OF PERSON SIGNING Vanderbilt University, 405 Kirkland Hall, Nashville,  
Tennessee 37240  
SIGNATURE Leona Marx DATE 11/28/94

Express Mail No. EL348123700US  
Attorney Docket No. 22000.0021U2  
PATENT

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APPLICATION  
FOR  
UNITED STATES LETTERS PATENT  
ON

**A NOVEL METHOD FOR IMPORTING BIOLOGICALLY  
ACTIVE MOLECULES INTO CELLS**

25

BY

**Yao-Zhong Lin**, a citizen of the People's Republic of China and **Jack J. Hawiger**, a citizen of the United States of America, residing respectively at 101 Stoneway Close, Nashville, Tennessee 37209 and 1818 Laurel Ridge Drive, 30 Nashville, Tennessee 37215 U.S.A.

# A NOVEL METHOD FOR IMPORTING BIOLOGICALLY ACTIVE MOLECULES INTO CELLS

## ACKNOWLEDGEMENTS

5

This invention was made with partial government support under NIH Grant Nos. HL 30648 and HL 45994 awarded by the National Heart, Lung and Blood Institute. The United States government has certain rights in the invention.

10

## BACKGROUND OF THE INVENTION

### FIELD OF THE INVENTION

15 The present invention relates generally to the delivery of biologically active molecules, such as peptides, polypeptides and nucleic acids, into the interior of cells. Specifically, the delivery is accomplished by administering to the cells a complex comprising the molecule linked to a signal peptide. Such delivery can be utilized for many purposes such as peptide 20 therapy, antisense oligonucleotide therapy and gene transfer.

### BACKGROUND ART

Peptides have been developed for many therapeutic uses. For 25 example, diseases currently targeted by new peptide drugs include heart conditions, cancers, endocrine disorders, neurological defects, respiratory conditions, allergies and autoimmune diseases. Although the manufacture of known therapeutic peptides can be achieved by known methods, i.e., classic synthetic techniques or recombinant genetic engineering, delivery of the peptides 30 into a cell has remained problematic, since they cannot readily cross biological membranes to enter cells. Thus, current methods include permeabilization of the cell membrane or microinjection into the cell. Both of these methods have

PCT/US2000/012560

serious drawbacks. Permeabilization of cells, e.g., by saponin, bacterial toxins, calcium phosphate, electroporation, etc., can only be practically useful for *ex vivo* methods, and these methods cause damage to the cells. Microinjection requires highly skilled technicians (thus limiting its use to a laboratory setting), it

5 physically damages the cells, and it has only limited applications as it cannot be used to treat, for example, a mass of cells or an entire tissue, because one cannot feasibly inject large numbers of cells.

Similarly, delivery of nucleic acids has been problematic. Methods

10 currently employed include the permeabilization described above, with the above-described drawbacks, as well as vector-based delivery, such as with viral vectors, and liposome-mediated delivery. However, viral vectors can present additional risks to a patient, and liposome techniques have not achieved satisfactorily high levels of delivery into cells.

15 Signal peptide sequences,<sup>1</sup> which share the common motif of hydrophobicity, mediate translocation of most intracellular secretory proteins across mammalian endoplasmic reticulum (ER) and prokaryotic plasma membranes through the putative protein-conducting channels.<sup>2-11</sup> Alternative models for secretory protein transport also support a role for the signal sequence in targeting proteins to membranes.<sup>12-15</sup>

Several types of signal sequence-mediated inside-out membrane translocation pathways have been proposed. The major model implies that the

25 proteins are transported across membranes through a hydrophilic protein-conducting channel formed by a number of membrane proteins.<sup>2-11</sup> In eukaryotes, newly synthesized proteins in the cytoplasm are targeted to the ER membrane by signal sequences that are recognized generally by the signal recognition particle (SRP) and its ER membrane receptors. This targeting step

30 is followed by the actual transfer of protein across the ER membrane and out of the cell through the putative protein-conducting channel (for recent reviews, see references 2-5). In bacteria, the transport of most proteins across the

cytoplasmic membrane also requires a similar protein-conducting channel.<sup>7-11</sup> On the other hand, signal peptides can interact strongly with lipids, supporting the proposal that the transport of some secretory proteins across cellular membranes may occur directly through the lipid bilayer in the absence of any 5 proteinaceous channels.<sup>14-15</sup>

Thus, though many attempts have been made to develop effective methods for importing biologically active molecules into cells, both *in vivo* and *in vitro*, none has proved to be entirely satisfactory. This problem affects a wide 10 variety of therapies. The solution of this problem would greatly expand treatments of diseases for which delivery of a therapeutic molecule would be beneficial. The present invention solves this long-felt, broad spectrum problem by providing a method of importing a biologically active molecule into a cell 15 using mechanisms naturally occurring in cells and thus avoiding damaging the target cells. Additionally, the present method can be used to import molecules into large numbers of cells, including organs. Thus, this versatile inventive method can be employed in numerous treatments of diseases and disorders.

#### SUMMARY OF THE INVENTION

20 The present invention provides a method of importing a biologically active molecule into a cell *ex vivo* comprising administering to the cell, under import conditions, a complex comprising the molecule linked to an importation competent signal peptide, thereby importing the molecule into the 25 cell. Molecules that can be delivered by this method can include, for example, peptides, polypeptides, proteins, nucleic acids, carbohydrates, lipids, glycolipids, and therapeutic agents.

30 The present invention further provides a method of importing a biologically active molecule into a cell in a subject comprising administering to the subject a complex comprising the molecule linked to an importation

competent signal peptide, thereby importing the molecule into the cell of the subject.

Additionally, the instant invention provides a method of importing  
5 a biologically active molecule into the nucleus of a cell in a subject comprising  
administering to the subject a complex comprising the molecule linked to an  
importation competent signal peptide and a nuclear localization peptide, thereby  
importing the molecule into the nucleus of the cell of the subject.

10 Further, the present invention provides a method of regulating  
growth of a cell in a subject comprising administering to the subject a complex  
comprising a growth regulatory peptide linked to an importation competent  
signal peptide to import the growth regulatory peptide into the cell of the  
subject, thereby regulating the growth of the cell.

15 The present invention additionally provides a method of inhibiting  
expression in a cell in a subject of a gene controlled by transcription factor NF-  
κB comprising administering to the subject a complex comprising an importation  
competent signal peptide linked to a nuclear localization peptide of an active  
20 subunit of NF-κB complex.

Also, the present invention provides a method of stimulating the  
immune system of a subject comprising administering to the subject a complex  
comprising an importation competent signal peptide linked to an antigenic  
25 peptide.

The instant invention also provides a complex comprising a  
biologically active molecule linked to an importation competent signal peptide  
and to a nuclear localization peptide.

30 The present invention further provides a complex comprising an  
importation competent signal peptide linked to a biologically active molecule

selected from the group consisting of a nucleic acid, a carbohydrate, a lipid, a glycolipid and a therapeutic agent.

The present invention also provides a method of screening signal  
5 peptides for the ability to effect the importation of a biologically active molecule  
into a cell comprising administering to the cell a complex comprising the  
molecule linked to the signal peptide and determining whether the molecule is  
imported into the cell, the presence of importation of the molecule indicating a  
signal peptide which can effect importation.

10

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a graphic representation of [<sup>3</sup>H] thymidine  
incorporation by NIH 3T3 cells stimulated with either (a) SA peptide, SA $\alpha$   
15 peptide, ANL peptide or SM peptide or (b) acidic Fibroblast Growth Factor  
(aFGF).

#### **DESCRIPTION OF THE PREFERRED EMBODIMENTS**

20 The present invention may be understood more readily by  
reference to the following detailed description of specific embodiments and the  
Examples and Figures included therein.

The present invention provides the discovery that importing  
25 exogenous biologically active molecules into intact cells can be engineered by  
forming a complex by attaching an importation competent signal peptide  
sequence to a selected biologically active molecule and administering the  
complex to the cell. The complex is then imported across the cell membrane by  
the cell. Thus, the present invention provides a method of importing a  
30 biologically active molecule into a cell *ex vivo* or *in vivo* comprising administering  
to the cell, under import conditions, a complex comprising the molecule linked

to an importation competent signal peptide, thereby importing the molecule into the cell.

As used herein, "biologically active molecule" includes any

5 molecule which, if imported into a cell, can have a biological effect. Naturally only those molecules which are of a size which can be imported into the cell are within the scope of the invention. However, since very large proteins (ranging from molecular weights of about 100,000 to around 1 million) are exported by cells (e.g., antibodies, fibrinogen, and macroglobulin), very large proteins can be  
10 imported into cells by this method. Therefore, size ranges for proteins from a few amino acids to around a thousand amino acids can be used. A preferable size range for proteins is from a few amino acids to about 250 amino acids. For any molecule, size ranges can be up to about a molecular weight of about 1 million, with a preferable size range being up to a molecular weight of about  
15 25,000, and an even more preferable size range being up to a molecular weight of about 3,000. In addition, only those molecules which can be linked to a signal peptide, either directly or indirectly, are within the scope of the invention. Likewise, the present invention requires that the complex is administered under suitable conditions for effective import into the cell.

20

Examples of biologically active molecules include proteins, polypeptides and peptides, which include functional domains of biologically active molecules, such as growth factors, enzymes, transcription factors, toxins, antigenic peptides (as for vaccines), antibodies, and antibody fragments.

25 Additional examples of biologically active molecules include nucleic acids, such as plasmids, coding DNA sequences, mRNAs and antisense RNA molecules, carbohydrates, lipids and glycolipids. Further examples of biologically active molecules include therapeutic agents, in particular those with a low cell membrane permeability. Some examples of these therapeutic agents include  
30 cancer drugs, such as Daunorubicin,<sup>26</sup> and toxic chemicals which, because of the lower dosage that can be administered by this method, can now be more safely administered.

A specific example of a biologically active molecule is the peptide comprising the nuclear localization sequence (NLS) of acidic fibroblast growth factor (aFGF), listed herein as SEQ ID NO:2. As demonstrated in the examples below, the NLS of aFGF, when linked to a signal peptide and transported into

5 cells (e.g., the entire peptide listed herein as SEQ ID NO:4), induces a mitogenic response in the cells. Another example of a biologically active molecule is the peptide comprising the NLS of transcription factor NF- $\kappa$ B subunit p50, listed herein as SEQ ID NO:10. As shown in the examples herein, when a peptide comprising the signal sequence of K-FGF and the NLS of

10 transcription factor NF- $\kappa$ B p50 subunit, this peptide (called SN50) being listed herein as SEQ ID NO:9, is transfected into cells having transcription factor NF- $\kappa$ B, the normal translocation of active NF- $\kappa$ B complex into the nucleus is inhibited. In this manner, cell growth can be inhibited by inhibiting the action of NF- $\kappa$ B and therefore inhibiting the expression of genes controlled by

15 transcription factor NF- $\kappa$ B.

Yet another example of a biologically active molecule is an antigenic peptide. Antigenic peptides can be administered to provide immunological protection when imported by cells involved in the immune

20 response. Other examples include immunosuppressive peptides (e.g., peptides that block autoreactive T cells, which peptides are known in the art). Numerous other examples will be apparent to the skilled artisan.

Suitable import conditions are exemplified herein and include cell

25 and complex temperature between about 18°C and about 42°C, with a preferred temperature being between about 22°C and about 37°C. For administration to a cell in a subject, the complex, once in the subject, will of course adjust to the subject's body temperature. For *ex vivo* administration, the complex can be administered by any standard methods that would maintain viability of the cells,

30 such as by adding it to culture medium (appropriate for the target cells) and adding this medium directly to the cells. As is known in the art, any medium used in this method can be aqueous and non-toxic so as not to render the cells

non-viable. In addition, it can contain standard nutrients for maintaining viability of cells, if desired. For *in vivo* administration, the complex can be added to, for example, a blood sample or a tissue sample from the patient, or to a pharmaceutically acceptable carrier, e.g., saline and buffered saline, and

5 5 administered by any of several means known in the art. Examples of administration include parenteral administration, e.g., by intravenous injection including regional perfusion through a blood vessel supplying the tissues(s) or organ(s) having the target cell(s), or by inhalation of an aerosol, subcutaneous or intramuscular injection, topical administration such as to skin wounds and

10 10 lesions, direct transfection into, e.g., bone marrow cells prepared for transplantation and subsequent transplantation into the subject, and direct transfection into an organ that is subsequently transplanted into the subject. Further administration methods include oral administration, particularly when the complex is encapsulated, or rectal administration, particularly when the

15 15 complex is in suppository form. A pharmaceutically acceptable carrier includes any material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected complex without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it

20 20 is contained.

Administration can be performed for a time length of about 1 minute to about 72 hours. Preferable time lengths are about 5 minutes to about 48 hours, and even more preferably about 5 minutes to about 20 hours, and

25 25 even more preferably about 5 minutes to about 2 hours. Optimal time lengths and conditions for any specific complex and any specific target cell can readily be determined, given the teachings herein and knowledge in the art.<sup>27</sup> Specifically, if a particular cell type *in vivo* is to be targeted, for example, by regional perfusion of an organ or tumor, cells from the target tissue can be

30 30 biopsied and optimal dosages for import of the complex into that tissue can be determined *in vitro*, as described herein and as known in the art, to optimize the *in vivo* dosage, including concentration and time length. Alternatively, culture

cells of the same cell type can also be used to optimize the dosage for the target cells *in vivo*.

For either *ex vivo* or *in vivo* use, the complex can be administered

5 at any effective concentration. An effective concentration is that amount that results in importation of the biologically active molecule into the cell. Such a concentration will typically be between about 0.5 nM to about 100  $\mu$ M (culture medium concentration (*ex vivo*) or blood serum concentration (*in vivo*)).

10 Optimal concentrations for a particular complex and/or a particular target cell can be readily determined following the teachings herein. Thus, *in vivo* dosages of the complex include those which will cause the blood serum concentration of the complex to be about 0.5 nM to about 100  $\mu$ M. A preferable concentration is about 2 nM to about 50  $\mu$ M. The amount of the complex administered will, of course, depend upon the subject being treated, the subject's age and weight,

15 the manner of administration, and the judgment of the skilled administrator. The exact amount of the complex will further depend upon the general condition of the subject, the severity of the disease/condition being treated by the administration and the particular complex chosen. However, an appropriate amount can be determined by one of ordinary skill in the art using routine

20 optimization given the teachings herein.

Parenteral administration, e.g., regional perfusion, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, such as liquid solutions, suspensions, or emulsions. A slow release or

25 sustained release system, such as disclosed in U.S. Patent No. 3,710,795, can also be used, allowing the maintenance of a constant level of dosage.

Depending on the intended mode of administration, the pharmaceutical compositions may be in the form of solid, semi-solid or liquid

30 dosage forms, such as, for example, tablets, suppositories, pills, capsules, powders, liquids, suspensions, lotions, creams, gels, or the like, preferably in unit dosage form suitable for single administration of a precise dosage. The

compositions will include, as noted above, an effective amount of the selected drug in combination with a pharmaceutically acceptable carrier and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc.

5

For solid compositions, conventional nontoxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talc, cellulose, glucose, sucrose, magnesium carbonate, and the like. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc. an active compound as described herein and optional pharmaceutical adjuvants in an excipient, such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington's Pharmaceutical Sciences*.<sup>27</sup>

20 The present invention utilizes a complex comprising the selected biologically active molecule linked to an importation competent signal peptide. As discussed above, the biologically active molecule can be selected from any of a variety of molecules, with its selection being dependent upon the purpose to be accomplished by importing the molecule into the selected cell. An  
25 25 “importation competent signal peptide,” as used herein, is a sequence of amino acids generally of a length of about 10 to about 50 or more amino acid residues, many (typically about 55-60%) residues of which are hydrophobic such that they have a hydrophobic, lipid-soluble portion.<sup>1</sup> The hydrophobic portion is a common, major motif of the signal peptide, and it is often a central part of the  
30 30 signal peptide of protein secreted from cells. A signal peptide is a peptide capable of penetrating through the cell membrane to allow the export of cellular proteins. The signal peptides of this invention, as discovered herein, are also

"importation competent," i.e., capable of penetrating through the cell membrane from outside the cell to the interior of the cell. The amino acid residues can be mutated and/or modified (i.e., to form mimetics) so long as the modifications do not affect the translocation-mediating function of the peptide. Thus the word

- 5 "peptide" includes mimetics and the word "amino acid" includes modified amino acids, as used herein, unusual amino acids, and D-form amino acids. All importation competent signal peptides encompassed by this invention have the function of mediating translocation across a cell membrane from outside the cell to the interior of the cell. Such importation competent signal peptides could
- 10 potentially be modified such that they lose the ability to export a protein but maintain the ability to import molecules into the cell. A putative signal peptide can easily be tested for this importation activity following the teachings provided herein, including testing for specificity for any selected cell type.

- 15 Signal peptides can be selected, for example, from the SIGPEP database, which also lists the origin of the signal peptide.<sup>30, 38</sup> When a specific cell type is to be targeted, a signal peptide used by that cell type can be chosen. For example, signal peptides encoded by a particular oncogene can be selected for use in targeting cells in which the oncogene is expressed. Additionally,
- 20 signal peptides endogenous to the cell type can be chosen for importing biologically active molecules into that cell type. And again, any selected signal peptide can be routinely tested for the ability to translocate across the cell membrane of any given cell type according to the teachings herein. Specifically, the signal peptide of choice can be conjugated to a biologically active molecule,
- 25 e.g., a functional domain of a cellular protein or a reporter construct, and administered to a cell, and the cell is subsequently screened for the presence of the active molecule.

- 30 The presence of modified amino acids in the signal peptide can additionally be useful for rendering a complex, wherein the biologically active molecule is a peptide, polypeptide or protein, more resistant to peptidases in the subject. Thus these signal peptides can allow for more effective treatment by

allowing more peptides to reach their target and by prolonging the life of the peptide before it is degraded. Additionally, one can modify the amino acid sequence of the signal peptide to alter any proteolytic cleavage site present in the original signal sequence for removing the signal sequence. Cleavage sites are 5 characterized by small, positively charged amino acids with no side chains and are localized within about 1 to about 4 amino acids from the carboxy end of the signal peptide.<sup>1</sup>

An example of a useful signal peptide is the signal peptide from 10 Kaposi fibroblast growth factor (K-FGF),<sup>16-17</sup> listed herein as SEQ ID NO:5. Any signal peptide, however, capable of translocating across the cell membrane into the interior of the selected target cell can be used according to this invention.

15 By "linked" as used herein is meant that the biologically active molecule is associated with the signal peptide in such a manner that when the signal peptide crosses the cell membrane, the molecule is also imported across the cell membrane. Examples of such means of linking include (1) when the molecule is a peptide, the signal peptide (and a nuclear localization peptide, if 20 desired) can be linked by a peptide bond, i.e., the two peptides can be synthesized contiguously; (2) when the molecule is a polypeptide or a protein (including antibody), the signal peptide (and a nuclear localization peptide, if desired) can be linked to the molecule by a peptide bond or by a non-peptide covalent bond (such as conjugating a signal peptide to a protein with a 25 crosslinking reagent); (3) for molecules that have a negative charge, such as nucleic acids, the molecule and the signal peptide (and a nuclear localization peptide, if desired) can be joined by charge-association between the negatively-charged molecule and the positively-charged amino acids in the peptide or by other types of association between nucleic acids and amino acids; (4) chemical 30 ligation methods can be employed to create a covalent bond between the carboxy-terminal amino acid of the signal peptide (and a nuclear localization

peptide, if desired) and the molecule. Methods (1) and (2) are typically preferred.

Examples of method (1) are shown below wherein a peptide is synthesized, by standard means known in the art,<sup>24,25</sup> that contains, in linear order from the amino-terminal end, a signal peptide sequence, an optional spacer amino acid region, and a biologically active amino acid sequence. Such a peptide could also be produced through recombinant DNA techniques, expressed from a recombinant construct encoding the above-described amino acids to create the peptide.<sup>28</sup>

For method (2), either a peptide bond, as above, can be utilized or a non-peptide covalent bond can be used to link the signal peptide with the biologically active polypeptide or protein. This non-peptide covalent bond can be formed by methods standard in the art, such as by conjugating the signal peptide to the polypeptide or protein via a crosslinking reagent, for example, glutaraldehyde. Such methods are standard in the art.<sup>29</sup> For method (3) the molecules can simply be mixed with the signal peptide and thus allowed to associate. These methods are performed in the same manner as association of nucleic acids with cationic liposomes.<sup>32-34</sup> Alternatively, covalent (thioester) bonds can be formed between nucleic acids and peptides. Such methods are standard in the art.

For method (4), standard chemical ligation methods, such as using chemical crosslinkers interacting with the carboxy-terminal amino acid of the signal peptide, can be utilized. Such methods are standard in the art (see, e.g., Goodfriend,<sup>31</sup> which uses water-soluble carbodiimide as a ligating reagent) and can readily be performed to link the carboxy terminal end of the signal peptide to any selected biologically active molecule.

30

The complex that is administered to a subject can further comprise a liposome. Cationic and anionic liposomes are contemplated by this invention,

as well as liposomes having neutral lipids. Cationic liposomes can be complexed with the signal peptide and a negatively-charged biologically active molecule by mixing these components and allowing them to charge-associate. Cationic liposomes are particularly useful when the biologically active molecule is a

5 nucleic acid because of the nucleic acid's negative charge. Examples of cationic liposomes include lipofectin, lipofectamine, lipofectace and DOTAP.<sup>32-34</sup> Anionic liposomes generally are utilized to encase within the liposome the substances to be delivered to the cell. Procedures for forming cationic liposomes encasing substances are standard in the art<sup>35</sup> and can readily be

10 utilized herein by one of ordinary skill in the art to encase the complex of this invention.

Any selected cell into which import of a biologically active molecule would be useful can be targeted by this method, as long as there is a

15 means to bring the complex in contact with the selected cell. Cells can be within a tissue or organ, for example, supplied by a blood vessel into which the complex is administered. Additionally, the cell can be targeted by, for example, inhalation of the molecule linked to the peptide to target the lung epithelium. Some examples of cells that can be targeted by this inventive method include

20 fibroblasts, epithelial cells, endothelial cells, blood cells and tumor cells, among many. In addition, the complex can be administered directly to a tissue site in the body. As discussed above, the signal peptide utilized can be chosen from signal peptides known to be utilized by the selected target cell, or a desired signal peptide can be tested for importing ability given the teachings herein.

25 Generally, however, all signal peptides have the common ability to cross cell membranes due, at least in part, to their hydrophobic character. Thus, in general, a membrane-permeable signal peptide can be designed and used for any cell type, since all eukaryotic cell membranes have a similar lipid bilayer.

30 One particularly useful example is to import an antigenic peptide into cells of the immune system, thereby allowing the antigen to be presented by antigen-presenting cells, and an immune response to the antigen to be developed

by the subject. These antigenic peptide-containing complexes can be administered to the subject according to standard methods of administering vaccines, e.g., intramuscularly, subcutaneously or orally, and effectiveness can be measured by subsequent measuring of the presence of antibodies to the antigen.

5

The present invention also provides a method of importing a biologically active molecule into the nucleus of a cell in a subject comprising administering to the subject a complex comprising the molecule linked to an importation competent signal peptide and a nuclear localization peptide, thereby 10 importing the molecule into the nucleus of the cell of the subject. A nuclear localization peptide, as used herein, is a peptide having the function of delivering an intracellular peptide into the nucleus of the cell. Such nuclear localization sequences are known in the art to have this function.<sup>36,37</sup> An example of a nuclear localization peptide is the nuclear localization sequence of 15 aFGF, listed herein as SEQ ID NO:2. An example of a signal peptide (K-FGF) linked to a nuclear localization peptide (aFGF) is set forth in SEQ ID NO:3. As these examples demonstrate, the nuclear localization peptide sequences can be synthesized as a peptide contiguous with the signal peptide, if desired. Additionally, separate peptides can be linked by any means such as described 20 herein.

The present invention also provides a method of regulating growth of a cell in a subject comprising administering to the subject a complex comprising a growth regulatory peptide linked to an importation competent 25 signal peptide to import the growth regulatory peptide into the cell of the subject, thereby regulating the growth of the cell. Growth can be stimulated or inhibited depending upon the growth regulatory peptide selected. It is to be noted that the present invention provides regulation of cell growth also by administering a nucleic acid encoding a growth regulatory peptide under 30 functional control of a suitable promoter for expression in a specific target cell, wherein the nucleic acid is complexed with a signal peptide and administered to the target cell.

There are numerous growth regulatory peptides known in the art, any of which can be utilized in this invention, if appropriate for the target cell type and the type of regulation desired. The signal peptide facilitates the efficient import of the growth regulatory peptide into the target cell and, once 5 the regulatory peptide is imported, it functions to regulate cell growth in its specific manner. A particularly useful target cell is a tumor cell in which the method can be used to inhibit further aberrant cell growth. Cell growth can be stimulated by administering a growth regulatory peptide comprising the nuclear localization sequence of acidic fibroblast growth factor (aFGF). Cell growth can 10 be inhibited by administering peptides that inhibit growth, for example peptides that inhibit transcription in the cell, such as the NLS of the p50 subunit of transcription factor NF- $\kappa$ B.

An example of this method is seen below in the examples wherein 15 the growth regulatory peptide stimulates cell growth and comprises the nuclear localization signal of aFGF. As this example demonstrates, the growth regulatory peptide, if desired, can be synthesized contiguously with the signal peptide, though any known method can be utilized to link them. An example of a contiguous peptide is set forth in SEQ ID NO:3 and SEQ ID NO:4. Another 20 example is provided below, wherein a complex (listed as SEQ ID NO:9) comprising the membrane-permeable signal peptide of K-FGF linked to the NLS of transcription factor NF- $\kappa$ B p50 subunit is administered and inhibits the expression of genes encoding pro-inflammatory mediators.

25 The invention also provides a method of inhibiting expression in a cell in a subject of a gene controlled by transcription factor NF- $\kappa$ B comprising administering to the subject a complex comprising an importation competent signal peptide linked to a nuclear localization peptide of an active subunit of NF- $\kappa$ B complex. Many genes controlled by NF- $\kappa$ B are known in the art, and 30 others can be readily tested by standard means. Examples of such genes include cytokines and interleukins, such as IL-1, IL-6, granular colony stimulating factor, plasminogen activator inhibitor and procoagulant tissue factor. Additionally,

organisms having genes affected by NF- $\kappa$ B can be inhibited by this method, such organisms including human immunodeficiency virus (HIV) and cytomegalovirus (CMV). The optimal inhibitory peptide for specific cell types and specific genes can readily be determined by standard methods given the teachings herein.

5 Additionally, the optimal inhibitory peptide for a specific cell type subjected to a specific stimulant can readily be determined.

An example is provided herein wherein translocation of the NF- $\kappa$ B complex to the nucleus in endothelial cells stimulated with lipopolysaccharide

10 (LPS) is inhibited by a complex comprising a signal peptide linked to the NLS of subunit p50 of NF- $\kappa$ B. Presumably, the NLS of subunit p50 interferes with translocation of the complex to the nucleus due to competitive binding. Any cell type subjected to any (or no) stimulus can be readily screened for the optimal inhibitory peptide, i.e., the optimal NLS of a subunit of NF- $\kappa$ B, for that cell type. For example, for LEII cells, as demonstrated herein, the NLS of p50 is optimal.

The subunits of NF- $\kappa$ B complex are known in the art.<sup>43</sup> They include p50, p65 and cellular REL (c-REL). The nuclear localization sequences

20 of these subunits are also known. An "active" subunit of NF- $\kappa$ B complex, as used herein, means a subunit which, when it is inhibited, causes transcription factor NF- $\kappa$ B not to function to mediate transcription of genes under its control. The nuclear localization peptide used in this method can be a modification of the known NLS of these subunits as long as it retains the function of inhibiting 25 expression of a gene controlled by NF- $\kappa$ B, as can be readily determined according to the teachings herein and knowledge in the art.

The invention further provides a method of stimulating the immune system of a subject comprising administering to the subject a complex 30 comprising an importation competent signal peptide linked to an antigenic peptide. The complex can be administered to the subject by standard means known in the art for administering vaccines. The method can facilitate uptake

of the antigen into cells for subsequent antigen presentation and the resultant known cascade of the immune system to result in the stimulation of immunity to the antigen.

5           Furthermore, if known peptides for blocking autoreactive T cells are linked to a signal peptide and administered to a subject, an immunosuppressive effect can be stimulated in the subject. Such a method of stimulating immunosuppression can be used to treat autoimmune diseases such as multiple sclerosis. These blocking peptides can also be administered by

10          known methods for administering peptides, such as methods for administering vaccines.

The invention also provides a complex comprising a biologically active molecule linked to an importation competent signal peptide and to a

15          nuclear localization peptide. The linkage can be made as described above or otherwise known in the art. Though, as described above, any signal peptide and any nuclear localization sequence can be utilized, such a complex is exemplified by the amino acid sequences set forth in SEQ ID NO:3 and SEQ ID NO:4, which contain the K-FGF signal peptide (SEQ ID NO:5) linked to the aFGF

20          nuclear localization peptide (SEQ ID NO:2).

The invention further provides a complex comprising an importation competent signal peptide linked to biologically active molecule selected from the group consisting of a nucleic acid, a carbohydrate, a lipid, a

25          glycolipid and a therapeutic agent. This complex can further comprise a liposome. These complexes can be formed as described above. Liposomes can be selected as described above. The complex can be placed in a pharmaceutically acceptable carrier.

30          As used herein, "a" can mean one or more, depending on the context in which it is used.

The invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

## 5 Statement Concerning Utility

The present method, which provides an effective method for importing biologically active molecules into cells, has many uses, both *in vivo* and *ex vivo*. Specific utilities using the method are apparent and are exemplified

10 as follows. *In vivo*, the method can be used to deliver into cells therapeutic molecules, such as peptides and proteins to regulate aberrant functions or to supply deficient cells; DNA for gene therapy (e.g., to provide the CFTR gene in cystic fibrosis patients); RNA for antisense therapy (e.g., to inhibit growth as in inhibiting expression in cancer cells); and therapeutic agents such as cancer

15 drugs or toxic chemicals (which can be administered in lower dosages with this method as compared to previous methods not utilizing a signal peptide to more efficiently enter the cells). *Ex vivo*, the method allows efficient transfection of cells without performing cell-damaging procedures. Therefore, this method is useful *ex vivo* in any method that utilizes transfection, such as transfecting

20 reporter genes into cells to screen for compounds that affect expression of the reporter gene, and for transfecting bone marrow cells, blood cells, cells of an organ for subsequent transplantation into a subject, or culture cells, with a gene to effect protein expression in the cells.

25 More specifically, this method can be used for anti-thrombotic therapy by administering functional domains of known cell receptors which mediate aggregation of platelets, by competitive binding. Additionally, the method can be used for immunosuppression in autoimmune diseases by introducing immunosuppressive peptides into cells involved in the immune

30 response. Furthermore, growth inhibitors can be administered by this method to tumor cells to treat, for example, cancer cells.

This method can also be used to facilitate the absorption of biologically active molecules from, e.g., the mouth, stomach or intestinal tract by facilitating movement of the molecules into the connective tissue beneath the lining of the digestive tract. Also, by allowing one to design signal peptides with 5 modified amino acids, one can stabilize biologically active peptides by making them more resistant to peptidases and therefore also prolong the action of the peptide.

## EXAMPLES

10 The peptides used herein were synthesized by a stepwise solid-phase peptide synthesis approach<sup>24</sup> and purified by high performance liquid chromatography using C<sub>18</sub> reverse phase column as described.<sup>25</sup> The exact molecular weights of the purified peptides were confirmed by mass spectrometry analysis.

15 Amino acid residues 1–16 of the SM peptide were patterned after the predicted signal peptide sequence of K-FGF<sup>16,17</sup> (listed separately herein as SEQ ID NO:5), residues 17–19 were designed as a spacer, and residues 20–26 contain an epitope tag recognized by antibody (see SEQ ID NO:1). Amino acid 20 residues 1–19 of the SA peptide are identical to those of the SM peptide. However, its carboxyl terminal residues 20–26 are same as the sequence of the ANL peptide (SEQ ID NO:2), which is derived from the nuclear localization sequence of acidic FGF.<sup>18</sup> The SA peptide is listed as SEQ ID NO:3. The amino acid sequence of the SA $\alpha$  peptide was the same as that of the SA peptide 25 except it had a two amino acid residue extension (Met-Pro) at the carboxyl terminus, which created an epitope (Leu-Met-Pro) for anti-SM peptide antibody. The SA $\alpha$  peptide is listed herein as SEQ ID NO:4.

### Membrane-Permeable Signal Sequence Peptide (SM Peptide)

30 A 26-residue peptide (referred to as SM, listed herein as SEQ ID NO:1) that contained the predicted signal sequence of Kaposi fibroblast growth

factor<sup>16,17</sup>(K-FGF) was chemically synthesized. An indirect immunofluorescence assay using antibody against epitope tag-containing SM peptide was employed to follow translocation of the SM peptide to the intracellular compartments of NIH 3T3 cells. A polyclonal anti-SM peptide antibody against the SM peptide-  
5 keyhole limpet haemocyanin conjugate (Pierce) was raised in rabbits and reacted with SM peptide in ELISA (titer > 1:30,000). The intracellular SM peptide was detected by an indirect immunofluorescence assay using affinity-purified anti-SM peptide IgG and rhodamine-labelled anti-rabbit antibody (Kirkegaard & Perry). Briefly, confluent NIH 3T3 cells on the chamber slides (Nunc) were treated with  
10 either 0.5 ml SM peptide solution (100  $\mu$ g ml<sup>-1</sup>) in DMEM containing 10% FBS or with 0.5 ml DMEM containing 10% FBS only for 30 minutes at 37°C. The cells were fixed with 3.5% paraformaldehyde solution in PBS followed by 0.25% Triton X-100 in PBS and then treated with 1:20 anti-SM peptide IgG in PBS containing 0.5% bovine serum albumin (BSA) for 1.5 h. The intracellular SM  
15 peptide-antibody complexes were visualized by subsequent incubation with a rhodamine-labelled anti-rabbit polyclonal antibody for 1 h. In control systems, anti-SM peptide antibody was preabsorbed with the SM peptide. Intracellular localization of the SA $\alpha$  peptide was detected by immunofluorescence assay using affinity-purified anti-SM peptide IgG as described above for SM peptide.  
20 Following incubation of cells with SM peptide, intracellular deposits were observed in almost all cells. A ten-step z-position sectional scanning of the SM peptide-treated cells by the confocal laser scanning microscopy (CLSM) affirmed that these deposits were intracellular. Immunodetection of the SM peptide was specific because cells incubated with the peptide-antibody complex showed no  
25 evidence of intracellular peptide. Likewise, cells not exposed to SM peptide or cells exposed to SM peptide followed by the secondary antibody alone were negative. If cells were fixed with paraformaldehyde before peptide treatment, the cellular import of SM peptide was prevented.  
30 To determine the rate of SM peptide import across cell membranes, a kinetic experiment was carried out with the SM peptide-treated NIH 3T3 cells. NIH 3T3 cells were treated at 37°C with 0.5 ml SM peptide at

100  $\mu\text{g mL}^{-1}$  in DMEM containing 10% FBS for 1, 5, 15, 30, 60 and 120 minutes. Intracellular SM peptide deposits were detected by indirect immunofluorescence assay as described above. The intracellular staining of intracellular SM peptide was observed during the first 5 minute interval and 5 plateaued at about 30 minutes, indicating that the signal sequence-mediated peptide import into cells is rapid. The 30-minute time point was therefore selected to determine the optimal peptide concentrations for detectable import.

To determine the optimal peptide concentration, NIH 3T3 cells 10 were treated for 30 minutes at 37°C with 0.5 ml SM peptide solution in DMEM containing 10% FBS at the following concentrations: 0, 2, 10, 50, 100, and 150  $\mu\text{g mL}^{-1}$ . Intracellular localization of the SM peptide was detected by indirect immunofluorescence assay as described above. Indirect immunofluorescence assay demonstrated detectable peptide in the form of intracellular punctate 15 deposits when the cells were exposed to 2  $\mu\text{g/ml}$  (about 800 nM) of peptide. The cellular import was concentration-dependent and reached a plateau between 50  $\mu\text{g/ml}$  and 100  $\mu\text{g/ml}$ .

Transport of the SM peptide across the cell membrane was 20 temperature-dependent. No immunofluorescence staining was observed when the cells were treated for 30 minutes with 100  $\mu\text{g/ml}$  of the peptide at 4°C, whereas, cells treated at either 22°C or 37°C showed numerous punctate deposits. Accordingly, cellular import of the SM peptide resumed when the incubation temperature shifted from 4°C to 37°C.

25

Moreover, this signal sequence-mediated import is not limited to NIH 3T3 cells. The intracellular localization of SM peptide has been observed in baby hamster kidney-21 cells, human umbilical vein endothelial cells (HUVECs) and rodent endothelial cell line (LE-II), by the above indirect 30 immunofluorescence assay, with the same results as with NIH 3T3 cells.

Membrane-Permeable Signal Peptide (SKP peptide)

Intracellular localization of the membrane-permeable peptide was also shown by treatment of cells with proteases following peptide import. For 5 this experiment, 41-residue peptide (referred to as SKP and listed herein as SEQ ID NO:6) that contained the same hydrophobic sequence as SM peptide followed by the sequence of K-FGF(129-153) was designed and synthesized. The latter was present in KP peptide not containing hydrophobic sequence that served as the control for the membrane-permeable SKP peptide. Both peptides 10 possess tyrosine residues, therefore they were radiolabeled with  $^{125}\text{I}$  and examined for their ability to translocate into intact NIH 3T3 cells, as described below. Substantial radioactivity was detected in the  $^{125}\text{I}$ -SKP peptide-treated cells but not in  $^{125}\text{I}$ -KP peptide-treated cells, indicating that the import of SKP peptide into cells was selectively achieved due to the presence of hydrophobic, 15 membrane-permeable sequence. The intracellular  $^{125}\text{I}$ -SKP peptide was resistant to the action of proteases. After treatment of cells containing  $^{125}\text{I}$ -SKP peptide with pronase or trypsin, no significant loss of cell-associated radioactivity was observed, suggesting that the  $^{125}\text{I}$ -SKP peptide was located in intracellular compartments (Table 1). The import of membrane-permeable peptide was not 20 dependent on ATP as high energy source because cells depleted of about 95% of ATP showed a similar  $^{125}\text{I}$ -SKP peptide uptake as compared to ATP-positive cells (Table 1).

Both SKP and KP peptides were radiolabeled with  $^{125}\text{I}$  by the 25 Iodogen method (Pierce). The specific activities of both peptides were similar ( $2.5 \times 10^4$  cpm/ng). NIH 3T3 cells were subcultured on a 60-mm dish and incubated at 37°C for 3 days. The confluent monolayers ( $1.6 \times 10^6$  cells) on each dish were then washed twice with PBS and treated with 15 ng of  $^{125}\text{I}$ -SKP or  $^{125}\text{I}$ -KP peptide at 37°C for the indicated time. The cells were washed eight 30 times with PBS and twice with 2 M NaCl buffer (pH 7.5) until no radioactivity could be detected in the washings. The washed cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 1 mM phenylmethylsulphonyl

fluoride, 1 mM dithiothreitol, and 1% Triton X-100) and the radioactivity in the cell lysates was counted in a Packard auto-gamma counter. In some experiments, the washed cells were further treated with pronase (1 mg/ml) or trypsin (0.05%) solution in DMEM for 5 min at 37°C. The supernatants and 5 cells were separated and their radioactivities were counted separately. For ATP depletion assay, cells were incubated with 5 $\mu$ g/ml antimycin, 6.5 mM 2-deoxyglucose, and 10 mM glucono- $\delta$ -lactone in DMEM for 2 h at 37°C before addition of  $^{125}$ I-SKP peptide. The levels of ATP in ATP-depleted cells and normal cells were determined by ATP bioluminescent assay kit (Sigma). No 10 measurable ATP was observed in ATP-depleted cells.

TABLE 1

15 **IMPORT OF  $^{125}$ I-SKP PEPTIDE INTO ATP-DEPLETED NIH 3T3 CELLS AND EFFECT OF PROTEASES ON CELL-ASSOCIATED  $^{125}$ I-SKP PEPTIDE**

<u>ATP Depletion</u>		<u>Counts in cells (cpm/1.6 x 10<sup>6</sup> cells)</u>
	Untreated cells	20,189 $\pm$ 2,109
20	APT-depleted cells	22,266 $\pm$ 3,602
<u>Protease Treatment</u>		<u>Counts in cell lysates</u>
	Untreated cells	21,323 $\pm$ 853
	Pronase	21,791 $\pm$ 1,953
	Trypsin	23,193 $\pm$ 310
		<u>Counts in supernatants</u>
		2,966 $\pm$ 838
		1,979 $\pm$ 75
		655 $\pm$ 70

25

For ATP depletion assay, confluent NIH 3T3 cells (1.6 x 10<sup>6</sup> cells) in each dish were treated with or without ATP depleting reagents (antimycin, 2-deoxyglucose, and glucono- $\delta$ -lactone) for 2 h at 37°C. Cells were then treated with 15 ng of  $^{125}$ I-SKP peptide for 30 min at 37°C. After complete removal of 30 extracellular  $^{125}$ I-labeled peptides, the radioactivity in the cell lysates was counted. For the assay using proteases, cells were treated with  $^{125}$ I-SKP peptide and washed as described above. The  $^{125}$ I-SKP peptide-associated cells were then treated with pronase (1 mg/ml), trypsin (0.05%), or diluent for 5 min at 37°C.

The radioactivities in the cell lysates and supernatants were counted separately. Data in Table 1 represent the mean the mean  $\pm$  SEM of triplicate determinations of a single experiment. The experiment was repeated three times with similar results.

5

Membrane-Permeable Signal Peptide with Functional Peptide Cargo (SA peptide)

Having demonstrated the feasibility of the cellular import of signal sequence-containing peptides, functional cargo in the form of a sequence responsible for the nuclear localization of cellular proteins was linked to a signal peptide. The nuclear localization sequence (NLS) of acidic FGF (aFGF), because it has previously been reported to play an essential role in aFGF mitogenic activity, was utilized.<sup>18</sup> It had previously been shown that a mutant aFGF with deletion in its NLS region Asn-Tyr-Lys-Lys-Pro-Lys-Leu (NYKKPKL), listed herein as SEQ ID NO:2, failed to stimulate DNA synthesis and cell proliferation *in vitro* although it could still bind to the FGF receptor and induce intracellular receptor-mediated tyrosine phosphorylation and c-fos expression.<sup>18</sup> Additionally, a recent study<sup>19</sup> of nuclear transport of aFGF suggested that translocation of aFGF to the nucleus was necessary for stimulating DNA synthesis by aFGF *in vitro*.

A 26-residue hybrid peptide (referred to as SA, listed herein as SEQ ID NO:3) was designed and synthesized. It contains the signal sequence of K-FGF<sup>16,17</sup> at its amino terminal region (residues 1–16 of SEQ ID NO:3) and a "functional cargo" in the form of a nuclear localization sequence (NLS) of aFGF<sup>18</sup> at its carboxyl terminal region (residues 20–26 of SEQ ID NO:3), separated by a spacer region of Ala-Ala-Ala (residues 17–19 of SEQ ID NO:3). Thus, the SA peptide differs from the SM peptide only in its 7-residue carboxyl terminal "cargo" region. A functional assay was performed in which SA peptide was able to induce a mitogenic response of NIH 3T3 cells measured by

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[<sup>3</sup>H]thymidine incorporation in a manner similar to aFGF bearing the same NLS.<sup>18</sup>

In this functional assay, confluent 3T3 cells grown initially in

5 DMEM containing 10% FBS were transferred to a low serum medium (DMEM containing 0.5% FBS) for 2 days. The test peptides, either SA peptide, SA $\alpha$  peptide, ANL peptide, or SM peptide, or aFGF, were added to a fresh low serum medium at the indicated concentrations at 37°C. After 16 hours, [<sup>3</sup>H]thymidine was added and 4 hours later, the cells were washed with PBS,

10 treated with trichloroacetic acid, solubilized with 0.15 M NaOH, and the radioactivity was determined in a liquid scintillation counter.

As shown in Fig. 1a, SA peptide stimulated [<sup>3</sup>H]thymidine incorporation 6-fold, while aFGF induced approximately an 8-fold stimulation in

15 the same assay (Fig. 1b). Bars represent the mean  $\pm$  S.E.M. of at least three independent experiments done in triplicate and calculated as multiplicity of counts in the tested sample over the control sample. SA peptide within the concentration range used (0 to 46  $\mu$ M) was not cytotoxic as determined by staining with fluorescein diacetate/ethidium bromide.<sup>20</sup>

20

#### Mitogenic Activity of the SA Peptide

To determine whether the mitogenic activity of SA peptide required its full length sequence, two control peptides were examined in the

25 same assay. They are the SM peptide containing signal peptide (listed herein as SEQ ID NO:1) and a 7-residue peptide (referred to as ANL, listed herein as SEQ ID NO:2) representing the NLS of aFGF. Neither control peptide showed any significant mitogenic activity when tested within comparable concentration ranges (Fig. 1a). These results suggest that neither the signal sequence alone

30 (SM peptide) nor the nuclear localization sequence alone (ANL peptide) was sufficient for mitogenesis. SA peptide therefore was effective in mitogenesis because it contained both the signal peptide sequence of K-FGF (for import

into the cell) and nuclear localization sequence of aFGF (for mitogenic activity).

To further confirm the mitogenic activity of the SA peptide, its effect on DNA synthesis was examined. Serum-starved NIH 3T3 cells were 5 treated with SA peptide, fixed, and the DNA concentration was determined by standard flow cytometric analysis. Specifically, confluent NIH 3T3 cells ( $1.3 \times 10^6$  cells) were serum-starved in DMEM containing 0.5% FBS for 2 days. The cells were untreated (control) or treated with SA peptide or aFGF for 20 h, harvested, spun down, and washed with serum-free PBS three times. The cells 10 were fixed with methanol precooled to -20°C for DNA analysis carried out by the Flow Cytometry Research Service of Vanderbilt University. The data were the mean  $\pm$  S.E.M. of six measurements and were analyzed for statistical significance by analysis of variance.

15 As shown in Table 2, the DNA synthesis in the S-phase of the cell cycle was significantly increased when the cells were treated with the SA peptide at  $100 \mu\text{g/ml}$ , which coincided with the active concentration in the thymidine incorporation assay (Fig. 1a). This result further confirms the role of the NLS region of aFGF in mitogenesis.<sup>18</sup> Thus, these data also are consistent with a 20 recent demonstration using a genetic approach that schwannoma-derived growth factor requires NLS to exert its mitogenic activity.<sup>21</sup>

TABLE 2

25 DNA SYNTHESIS STIMULATED BY SA PEPTIDE AS COMPARED TO aFGF		
	<u>Stimulus</u>	<u>Diploid % S Phase</u>
	Control	$7.2 \pm 0.7$
	SA ( $50 \mu\text{g ml}^{-1}$ )	$6.7 \pm 0.9$
	SA ( $100 \mu\text{g ml}^{-1}$ )	$13.1 \pm 0.5$ ( $P < 0.05$ )
30	aFGF ( $15 \text{ ng ml}^{-1}$ )	$27.8 \pm 2.3$ ( $P < 0.05$ )

However, compared with aFGF, the SA peptide is less mitogenically potent in both thymidine incorporation and DNA analysis assays (Fig. 1 and Table 2). aFGF binds to the FGF receptors on NIH 3T3 cells and induces the tyrosine phosphorylation of a number of intracellular proteins that have been suggested as the FGF receptor signalling substrates.<sup>22,23</sup> In contrast, SA peptide did not stimulate the tyrosine phosphorylation of these intracellular proteins in the same cells even at the concentrations sufficient to induce DNA synthesis. Taken together, these results, make it unlikely that the mitogenic effect of SA peptide was mediated by FGF receptors.

10

### Immunofluorescence Assay for Modified SA Peptide

The intracellular SA peptide could not be tracked by an immunofluorescence assay because it was not recognized by the available anti-SM peptide antibody. However, attaching two extra amino acid residues (Met-Pro) to the carboxyl terminus of the SA peptide produced a modified SA peptide (referred to as SA $\alpha$ , listed herein as SEQ ID NO:4) that contained a 3-amino acid epitope tag, Leu-Met-Pro, recognized by anti-SM peptide antibody in ELISA. Accordingly, intracellular SA $\alpha$  peptide was observed in a punctate staining pattern in the SA $\alpha$ -treated NIH 3T3 cells by an indirect immunofluorescence assay using anti-SM peptide antibody. Like SA peptide, SA $\alpha$  peptide was mitogenic in the thymidine incorporation assay. These results are consistent with the relationship between the SA peptides' import into the intracellular compartments and their mitogenic activity.

25

## Membrane-Permeable Signal Peptide with Functional Peptide Cargo (SN50)

Having demonstrated the feasibility of the cellular import of membrane-permeable SM and SKP peptides, another functional cargo was attached to the amino-terminal hydrophobic sequence conferring membrane-permeable capacity. For this purpose a sequence representing a functional domain of the nuclear factor  $\kappa$ B (NF- $\kappa$ B) responsible for a nuclear localization

signal was selected. Import of such a peptide into the cell would be measured by inhibition of nuclear translocation of NF- $\kappa$ B complex in stimulated cells. The NF- $\kappa$ B is a pleiotropic activator<sup>39,40</sup> that plays a critical role in the regulation of a number of cellular and viral genes, including the enhancer of human immunodeficiency virus (HIV). The inactive cytosolic form of NF- $\kappa$ B is a heterotrimer including p50, p65 and an inhibitory protein I $\kappa$ B.<sup>41,42</sup> Upon activation of cells with stimuli such as lipopolysaccharide (LPS) or cytokines,<sup>43,44,45</sup> I $\kappa$ B dissociates from the complex. This dissociation allows the translocation of heterodimer of p50 and p65 subunits to the nucleus. Both p50 and p65 subunits contain NLS, suggesting that the NLS sequence may be important for nuclear uptake of NF- $\kappa$ B.

To determine the functional significance of the NLS of p50 and p65 subunits, two peptides were designed and synthesized containing these sequence motifs. The first peptide (referred to as SN50, listed herein as SEQ ID NO:9) contained the signal sequence of K-FGF<sup>16,17</sup> at its amino-terminal region (residues 1-16) and a "functional cargo" in the form of NLS of NF- $\kappa$ B p50 subunit at its carboxy-terminal region (residues 17-26). The second peptide is also a 26-residue peptide (referred to as SN65, listed herein as SEQ ID NO:8) *seq (10/21)* that contains the same hydrophobic sequence and the NLS of p65 subunit. Both peptides were tested for their inhibitory effects on the nuclear translocation of the NF- $\kappa$ B complex in LE-II cells. Inducible  $\kappa$ B binding activity was detectable by electrophoretic mobility shift assay in nuclear extracts from cells treated with LPS for 1 h.<sup>43</sup> However, this LPS-induced  $\kappa$ B binding activity in nuclear fraction was reduced substantially in the SN50 peptide-treated cells. The inhibition by SN50 peptide was concentration-dependent, reading an 88% inhibition at 50  $\mu$ g/ml. In contrast, no inhibition was observed in SN65 peptide-treated LEII cells. To exclude the possibility that the inhibition was caused by the interference of SN50 peptide in the binding of oligonucleotide probe to the NF- $\kappa$ B complex, SN50 peptide was incubated *in vitro* with nuclear extracts and radio-labeled probe. This maneuver was without any measurable effect on LPS-induced  $\kappa$ B binding activity, suggesting that inhibition by SN50 peptide resulted

from its action at the stage in which the active NF- $\kappa$ B complex moves from cytosol to nucleus. To determine whether the inhibition by SN50 peptide required a hydrophobic, membrane-permeable sequence, two control peptides (SM and N50 peptides, listed herein as SEQ ID NOS: 1 and 10, respectively) 5 were also tested in the same mobility shift assay. N50 peptide contained the NLS without the hydrophobic sequence, whereas SM peptide contained a hydrophobic sequence without the NLS. Neither of these two peptides showed any significant effect on LPS-induced intracellular translocation of the NF- $\kappa$ B complex from the cytosol to the nucleus. These results suggest that neither the 10 hydrophobic sequence alone (SM peptide) nor the nuclear localization sequence alone (N50 peptide) was sufficient for causing a functional inhibition of the NF- $\kappa$ B. Therefore, the observed inhibitory effect of SN50 must be attributed to its intracellular import, which allowed the interaction of its intrinsic NLS with the nuclear membranes.

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SN50 peptide contained the same epitope tag as SM peptide and thus could be recognized by the anti-SM peptide antibody in ELISA. This allowed direct affirmation by an indirect immunofluorescence assay that SN50 peptide was imported into LE-II cells to exert its functional role. Results 20 showed that the intracellular SN50 peptide was distributed in a more nuclear staining pattern as compared to the intracellular SM peptide.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated 25 by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such 30 details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

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## REFERENCES

1. von Heijne, *J. Membrane Biol.* **115**:195-201 (1990).
2. Rapoport, *Science* **258**:931-936 (1992).
3. Gilmore, *Cell* **75**:589-592 (1993).
4. Sanders and Schekman, *J. Biol. Chem.* **267**:13791-13794 (1992).
5. Nunnari and Walter, *Curr. Opin. Cell Biol.* **4**:573-580 (1992).
6. Simon and Blobel, *Cell* **65**:371-380 (1991).
7. Poritz *et al.*, *Science* **250**:1111-1117 (1990).
8. Ribes *et al.*, *Cell* **63**:591-600 (1990).
9. Luirink *et al.*, *Nature* **359**:741-743 (1992).
10. Phillips and Sihavy, *Nature* **359**:744-746 (1992).
11. Simon and Blobel, *Cell* **69**:677-684 (1992).
12. Cobet *et al.*, *J. Biol. Chem.* **264**:10169-10176 (1989).
13. Zimmermann, *et al.*, *Biochimie* **72**:95-101 (1990).
14. Wickner, *Biochemistry* **27**:1081-1086 (1988).
15. Killian *et al.*, *EMBO J.* **9**:815-819 (1990).
16. Delli Bovi *et al.*, *Cell* **50**:729-737 (1987).
17. Taira *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**:2980-2984 (1987).
18. Imamura *et al.*, *Science* **249**:1567-1570 (1990).
19. Imamura and Mitsui, *J. Biol. Chem.* **267**:5676-5679 (1992).
20. Kajstura and Reiss, *K. Folia Histochem. Cytobiol.* **27**:39-48 (1989).
21. Kimura, *Proc. Natl. Acad. Sci. U.S.A.* **90**:2165-2169 (1993).
22. Coughlin *et al.*, *J. Biol. Chem.* **263**:988-993 (1988).
23. Friesel *et al.*, *Molec. Cell. Biol.* **9**:1857-1865 (1989).
24. Merrifield, *J. Am. Chem. Soc.* **85**:2149-2154 (1963).

25. Lin *et al.*, *Biochemistry* 27:5640-5645 (1988).
26. *Physician's Desk Reference*, 47th Ed., Medicinal Economics Data, Montvale, N.J., p. 2555 (1993).
27. *Remington's Pharmaceutical Sciences*, 18th Ed., E. W. Martin (ed.), Mack Publishing Co., Easton, Pennsylvania (1990).
28. Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).
29. Walter *et al.*, *Proc. Natl. Acad. Sci. USA* 77:5197 (1980).
30. von Heijne, *Protein Sequence Data Analysis* Vol. 1:41-42 (1987).
31. Goodfriend *et al.*, *Science* 143:1344 (1964).
32. Hawley-Nelson *et al.*, *Focus* 15(3):73-83 (1992).
33. Felgner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 84:7413 (1987).
34. Stewart *et al.*, *Human Gene Therapy* 3:267-275 (1992).
35. Nicolau *et al.*, *Methods Enzymol.* 149:157 (1987).
36. Adam, S.A. *et al.*, *Nature* 337:276-279 (1989).
37. Goldfarb, D.S. *et al.*, *Nature* 322:641-644 (1986).
38. von Heijne and Abrahmsen, L., *FEBS Letters* 224:439-446 (1989).
39. Lenardo, M.J. and Baltimore, D., *Cell* 58:227 (1989).
40. Baeuerle, P.A., and Baltimore, D., *Mol. Aspects Cell. Regul.* 6:409 (1991).
41. Baeuerle, P.A. and Baltimore, D., *Cell* 53:211 (1988).
42. Baeuerle, P.A. and Baltimore, D., *Science* 242:540 (1988).
43. Cordle, S.R. *et al.*, *J. Biol. Chem.* 268:11803 (1993).
44. Sen, R. and Baltimore, D., *Cell* 46:705 (1986).
45. Liberman, T.A. and Baltimore, D., *Mol. Cell. Biol.* 10:2327 (1990).

DRAFTED: 07/07/96

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Lin, Yao-Zhong  
Hawiger, Jack J.
- (ii) TITLE OF INVENTION: A NOVEL METHOD FOR IMPORTING  
BIOLOGICALLY ACTIVE MOLECULES INTO CELLS
- (iii) NUMBER OF SEQUENCES: 11
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  - (C) CITY: Atlanta
  - (D) STATE: Georgia
  - (E) COUNTRY: USA
  - (F) ZIP: 30303-1811
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Perryman, David G.
  - (B) REGISTRATION NUMBER: 33,438
  - (C) REFERENCE/DOCKET NUMBER: 2200.021
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 404/688-0770
  - (B) TELEFAX: 404/688-9880

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..16
- (D) OTHER INFORMATION: /label= a  
/note= "Signal peptide amino acid sequence of K-FGF"

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 17..19
- (D) OTHER INFORMATION: /label= b  
/note= "Spacer region"

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 20..26
- (D) OTHER INFORMATION: /label= c  
/note= "Epitope tag"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro  
1 5 10 15

Ala Ala Ala Asp Gln Asn Gln Leu Met Pro  
20 25

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..7
- (D) OTHER INFORMATION: /label= a  
/note= "Nuclear localization sequence of aFGF"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asn Tyr Lys Lys Pro Lys Leu  
1 5

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..16
- (D) OTHER INFORMATION: /label= a  
/note= "Signal peptide amino acid sequence of K-FGF"

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 17..19
- (D) OTHER INFORMATION: /label= b  
/note= "Spacer region"

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 20..26
- (D) OTHER INFORMATION: /label= c  
/note= "Nuclear localization sequence of aFGF",

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro  
1 5 10 15

Ala Ala Ala Asn Tyr Lys Lys Pro Lys Leu  
20 25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..16
- (D) OTHER INFORMATION: /label= a  
/note= "Signal peptide amino acid sequence of K-FGF"

(ix) FEATURE:

- (A) NAME/KEY: Peptide

- (B) LOCATION: 17..19
- (D) OTHER INFORMATION: /label= b  
/note= "Spacer region"

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 20..26
- (D) OTHER INFORMATION: /label= c  
/note= "Nuclear localization sequence of aFGF"

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 26..28
- (D) OTHER INFORMATION: /label= d  
/note= "Epitope tag"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala	Ala	Val	Ala	Leu	Leu	Pro	Ala	Val	Leu	Leu	Ala	Leu	Leu	Ala	Pro	
1				5					10						15	
Ala Ala Ala Asn Tyr Lys Lys Pro Lys Leu Met Pro																
								20	25							

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 16 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..16
- (D) OTHER INFORMATION: /label= a  
/note= "Signal peptide of K-FGF"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala	Ala	Val	Ala	Leu	Leu	Pro	Ala	Val	Leu	Leu	Ala	Leu	Leu	Ala	Pro
1				5					10						15

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 41 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro  
1 5 10 15

Glu Ile Leu Leu Pro Asn Asn Tyr Asn Ala Tyr Glu Ser Tyr Lys Tyr  
20 25 30

Pro Gly Met Phe Ile Ala Leu Ser Lys  
35 40

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Glu Ile Leu Leu Pro Asn Asn Tyr Asn Ala Tyr Glu Ser Tyr Lys Tyr  
1 5 10 15

Pro Gly Met Phe Ile Ala Leu Ser Lys  
20 25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro  
1 5 10 15

Ile Glu Glu Lys Arg Lys Arg Thr Tyr Glu  
20 25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro  
1 5 10 15

Val Asn Arg Lys Arg Asn Lys Leu Met Pro  
20 25

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Asn Arg Lys Arg Asn Lys Leu Met Pro  
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ile Glu Glu Lys Arg Lys Arg Thr Tyr Glu  
1 5 10

What is claimed is:

1. A method of importing a biologically active molecule into a cell *ex vivo* comprising administering to the cell a complex comprising the molecule linked to an importation competent signal peptide, thereby importing the molecule into the cell.
2. The method of Claim 1, wherein the administration is performed at about 18°C to about 42°C.
3. The method of Claim 1, wherein the molecule is selected from the group consisting of a peptide, polypeptide, and protein.
4. The method of Claim 1, wherein the molecule is selected from the group consisting of a nucleic acid, carbohydrate, lipid, glycolipid and therapeutic agent.
5. The method of Claim 1, wherein the signal peptide comprises the amino acid sequence set forth in SEQ ID NO:5.
6. A method of importing a biologically active molecule into a cell in a subject comprising administering to the subject a complex comprising the molecule linked to an importation competent signal peptide, thereby importing the molecule into the cell of the subject.
7. The method of Claim 6, wherein the molecule is selected from the group consisting of a peptide, polypeptide, and protein.
8. The method of Claim 6, wherein the molecule is selected from the group consisting of a nucleic acid, carbohydrate, lipid, glycolipid and therapeutic agent.
9. The method of Claim 6, wherein the signal peptide comprises the amino acid sequence set forth in SEQ ID NO:5.

10. The method of Claim 6, wherein the molecule is an antigenic peptide.
11. A method of importing a biologically active molecule into the nucleus of a cell in a subject comprising administering to the subject a complex comprising the molecule linked to an importation competent signal peptide and a nuclear localization peptide, thereby importing the molecule into the nucleus of the cell of the subject.
12. The method of Claim 11, wherein the signal peptide comprises the amino acid sequence set forth in SEQ ID NO:5.
13. The method of Claim 11, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO:2.
14. The method of Claim 11, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO:10.
15. The method of Claim 11, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO:11.
16. A method of regulating the growth of a cell in a subject comprising administering to the subject a complex comprising a growth regulatory peptide linked to an importation competent signal peptide, thereby regulating the growth of the cell in the subject.
17. The method of Claim 16, wherein the cell is a tumor cell.
18. The method of Claim 16, wherein the growth regulatory peptide stimulates the cell growth and comprises the nuclear localization sequence of acidic fibroblast growth factor.

19. The method of Claim 18, wherein the growth regulatory peptide comprises the amino acid sequence set forth in SEQ ID NO:3.

20. The method of Claim 18, wherein the growth regulatory peptide comprises the amino acid sequence set forth in SEQ ID NO:4.

21. The method of Claim 16, wherein the growth regulatory peptide inhibits the cell growth.

22. The method of Claim 21, wherein the growth regulatory peptide comprises the amino acid sequence set forth in SEQ ID NO:9.

23. A method of inhibiting expression in a cell in a subject of a gene controlled by transcription factor NF- $\kappa$ B comprising administering to the subject a complex comprising an importation competent signal peptide linked to a nuclear localization peptide of an active subunit of NF- $\kappa$ B complex.

24. The method of Claim 23, wherein the subunit of NF- $\kappa$ B is subunit p50.

25. The method of Claim 24, wherein the complex comprises the amino acid sequence set forth in SEQ ID NO:9.

26. A method of stimulating the immune system of a subject comprising administering to the subject a complex comprising an importation competent signal peptide linked to an antigenic peptide.

27. A complex comprising a biologically active molecule linked to an importation competent signal peptide and to a nuclear localization peptide.

28. The complex of Claim 27, wherein the signal peptide comprises the amino acid sequence set forth in SEQ ID NO 5.

29. The complex of Claim 27, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO 2.

30. The complex of Claim 27, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO 10.

31. The complex of Claim 27, wherein the nuclear localization peptide comprises the amino acid sequence set forth in SEQ ID NO 11.

32. A complex comprising an importation competent signal peptide linked to a biologically active molecule selected from the group consisting of a nucleic acid, a carbohydrate, a lipid, a glycolipid and a therapeutic agent.

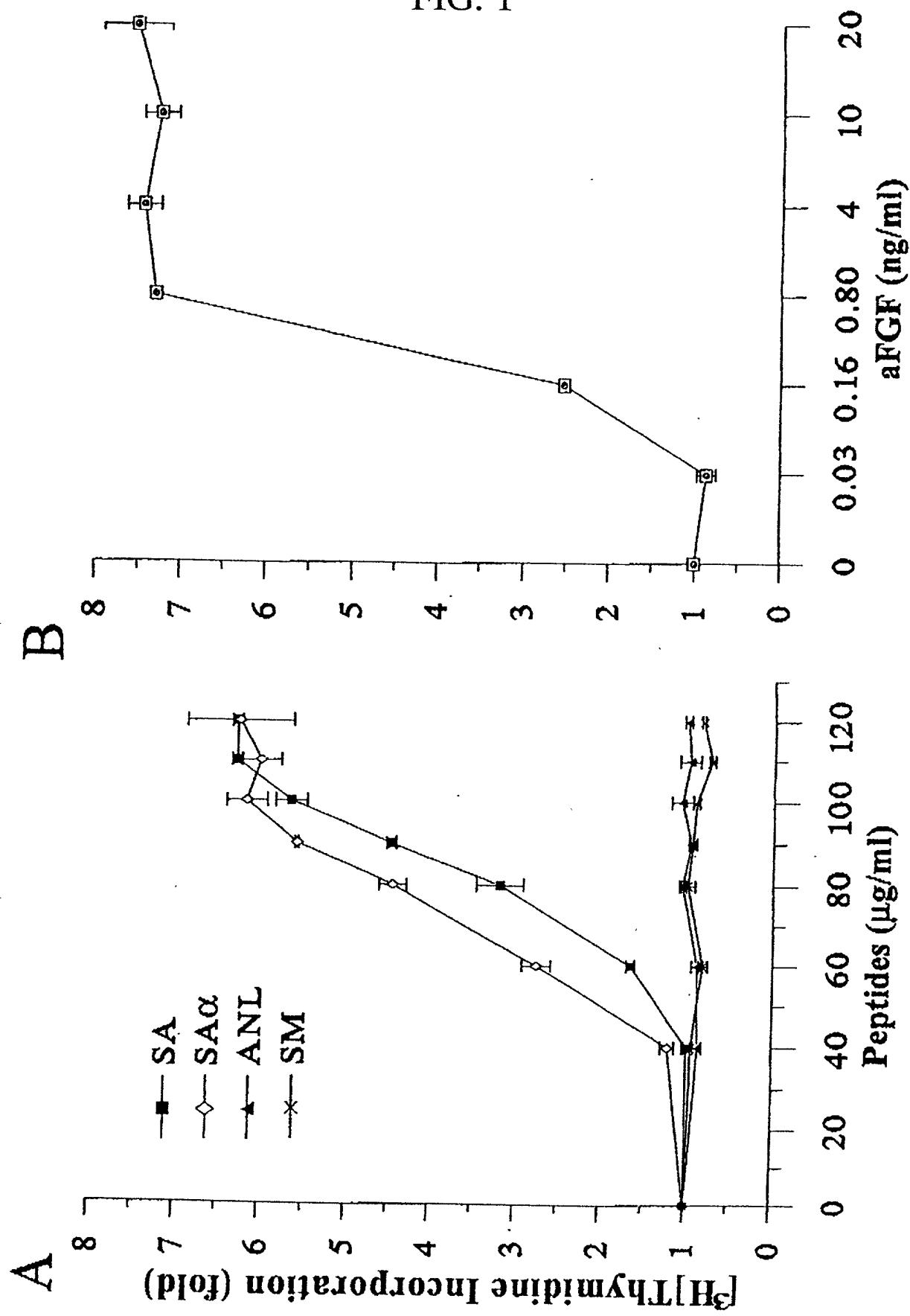
33. A method of screening signal peptides for the ability to effect the importation of a biologically active molecule into a cell comprising administering to the cell a complex comprising the molecule linked to the signal peptide and determining whether the molecule is imported into the cell, the presence of importation of the molecule indicating a signal peptide which can effect importation.

**ABSTRACT OF THE DISCLOSURE**

The present invention relates to the delivery of biologically active molecules, such as peptides, nucleic acids and therapeutic agents, into the interior of cells by administering to the cells a complex comprising the molecule linked to an importation competent signal peptide. Such delivery can be utilized for purposes such as peptide therapy, gene transfer, and antisense therapy.

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FIG. 1



-1-

Attorney Docket No. 2200.021

## DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

 Original       Supplemental       Substitute       PCT

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled "A NOVEL METHOD FOR IMPORTING BIOLOGICALLY ACTIVE MOLECULES INTO CELLS", which is described and claimed in the specification

(check one)

which is attached hereto, or  
 which was filed on June 13, 1994, as Application Serial No. 08/258,852 and with amendments through (if applicable), or  
 in International Application No. PCT/, filed , and as amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known by me to be material to the patentability of the claims of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate relating to this subject matter having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATIONS: (ENTER BELOW IF APPLICABLE)			PRIORITY CLAIMED (MARK APPROPRIATE BOX BELOW)	
APP. NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	YES	NO
N/A				

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information known by me to be material to the patentability of the claims of this application as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

-2-

APPLICATION SERIAL NO.	FILING DATE	STATUS (MARK APPROVAL, PENDING, EXPIRED)
PATENTED		PENDING
N/A		

I hereby appoint the following attorneys and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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